**A Critical (Re)evaluation of the Shroud of Turin Blood Data: Strength of Evidence in the Characterization of the Bloodstains**

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I'd like to begin with this quote from Dr. Alan Adler. This is from the very last interview that Heller & Adler did together in 1995: "The fact is, it [the blood on the Shroud] meets the descriptions that were required by the medical people, as to what it should look like. But the most interesting thing is now there is immunological evidence that it is primate blood." These sentences, particularly the last two, are what especially caught my eye regarding the science of the Shroud. My own research background is in the fields of immunology and cell biology and that's the main context in which I'd like to present the discussion today.

When it comes to blood on the Shroud, I believe that there are three main questions that most people have: First, is blood really present? Is the red stuff blood, if it's not, then what is it? Second, what kind of blood is it? Is it human blood or animal blood? What types of tests would you do to distinguish between these possibilities? And finally, whose blood is it? Is the blood consistent with being from a human male? Is the blood consistent with being from a single individual? Is it possible to extract information from the bloodstains that could tell you exactly who the blood may have belonged to? These are the types of issues that we'll discuss as we go through the presentation.

**First-round (presumptive) tests to evaluate the presence of blood**

What I would like to do is to first very briefly talk about so-called first-round types of tests, also known as presumptive type testing, involving various methods that evaluate the possibility that blood may be present. Then we'll move into the main portion of the talk and discuss immunological and molecular biology (DNA) testing. In those sections in particular, I'll be emphasizing the details of the methods that were used, because I believe that's important in helping to evaluate the significance of the results.

Regarding first-round type of tests, there are various ways to do this, but all of these essentially center around one thing: the detection of hemoglobin. Hemoglobin is the molecule that allows red blood cells to carry oxygen throughout the body and is a major signature of bloodstains. To put this in context, a single red blood cell contains approximately 250 million hemoglobin molecules. For fresh blood, this is relatively straightforward, investigators take advantage of the fact that hemoglobin has oxidase activity, which results in a specific color change of a substrate molecule. For aged blood, such tests can be somewhat problematic in that false positives may result as hemoglobin is not the

only molecule that possesses oxidase activity. False negatives may occur if the sample does not adequately go into solution (is not dissolved well). Because of such issues, related type tests were done on Shroud samples where the functional group of hemoglobin, heme, was chemically extracted and its fluorescence evaluated. Another type of chemical method that may be used is to mix reagents with the sample and look for crystal formation; this is a type of chemical test that has its ultimate readout using the microscope. Another method is spectroscopy, which involves exposing the sample to various wavelengths of light and measuring its relative transmission or absorbance. In the early 1970s, Frache and colleagues and Baima Bollone and coworkers would report negative results on Shroud samples using such methods. They did not conclude that blood was not present, rather, their conclusion was that blood could not be detected. Baima Bollone would revamp his solubilization methods and several years later (in the mod 1980s) report positive findings for hemoglobin using chemical methods, as would Heller and Adler in the same time period. In the mid-1980s, Heller and Adler would report positive findings for hemoglobin using spectroscopy as would Baraldi in 2008. In addition, Heller and Adler reported the detection of breakdown products of hemoglobin, biverdin and bilirubin; bilirubin was additionally detected using chemical methods. Garza-Valdes would report the detection of hemoglobin using immunochemical methods in the late 1990s, which I'll discuss in just a few minutes.

**Evaluation of non-heme constituents in bloodstains**

From these types of first-round (presumptive) tests, investigators will then move to evaluate the presence of non-heme constituents in the blood. This may be done using immunological methods, which can tell if the blood is human or animal, as well as provide information regarding ABO blood type. DNA methods may also be used, which can tell you all of the above, plus if the blood is consistent with being from a male or female.

Blood essentially consists of two fractions, a cellular fraction and a liquid fraction (serum). Tests for non-heme constituents involve the detection of albumin, which comprises approximately 60% of total serum protein, and immunoglobin (antibody), which is just under 20% of total serum protein. We'll look at albumin first. For these studies, purified human albumin was injected into rabbits to create antibodies specific for albumin. Such antibodies are then typically labeled with either a fluorescent or chemical tag that allows you to visualize their presence in an experimental system.

When white (control) Shroud fibers (without bloodstains) and bloodstained Shroud fibers were reacted with such antibodies, control fibers were negative whereas bloodstained fibers were positive. Taken together, these results suggest that albumin is present in bloodstained fibers of the Shroud. Heller and Adler also performed chemical tests to

verify the presence of albumin in bloodstained fibers, reported in the early 1980s.

Similar experiments were done to look for serum immunoglobulin, or antibody. (Antibody and immunoglobulin are the same thing). As previously discussed, antibodies may be used as tools, but they are also present in our serum, as a functional part of the immune system. In the human, there are five specific classes of immunoglobulin present (IgM, IgG, IgD, IgA, IgE). Similar to the albumin studies, these results showed that control fibers were negative for reactivity with anti-immunoglobulin, whereas bloodstained fibers gave a positive result. Importantly, in these studies irrelevant, nonspecific control antibodies, not directed against any blood components, were included, demonstrating that antibody binding was specific. Detection of serum immunoglobulin was reported both by Baima Bollone and Heller and Adler in the early 1980s. Baima Bollone would extend these findings by demonstrating that a particular subclass of antibody, IgG, could also be detected.

**What type of blood is present on the Shroud?**

As far as what type of blood it is, the antibodies that were used were raised against human proteins. However, this does not guarantee that this is the only type of species such antibodies will react against. This is referred to as cross-reactivity. Heller and Alder examined cross-reactivity directly in a set of parallel studies, not involving Shroud samples. They found that such "anti-human" antibodies reacted very well with purified human albumin, as expected, as this was what the antibody was raised against. However, they also observed very good reactivity with chimp albumin, and good reactivity with albumin purified from baboon. When you move further out to more unrelated species, such as cow or horse, here the antibody reactivity falls off. This is why Adler would conclude that these results demonstrate that primate blood is present. He would not extend this conclusion beyond that as cross-reactivity precludes a definitive answer (human blood) from these results.

It's also important to note that while studies have examined the possibility that human (primate) blood may be present, technically it is unknown if blood from other animal types may also exist. This is a blind spot in all of the studies that have been performed. To do this, one would have to use antibodies specific for albumin from various species (cow, chicken, goat, etc.) and test Shroud fibers directly for their reactivity. So, as far as what type of blood is it, the best, most scientifically objective conclusion is that primate blood has been detected. Or even more conservatively, to state that primate blood components are present.

**ABO Blood Typing and the Shroud**

Next, we'll talk about ABO blood typing; this can be performed on either fraction of the blood, the serum or cellular portion. A method

known as forward typing evaluates ABO molecules present on the surfaces of red blood cells. The reverse typing technique measures the presence or absence of specific antibodies that exist in the serum. We'll talk about both of these, and start with forward typing. Again, to put this into context, a single red blood cell expresses approximately two million ABO molecules on its surface. The particular blood type that an individual possesses is determined by the molecules that are present on their red blood cell surface. Type A individuals express A molecules, type B individuals, B molecules; Persons that are AB express both A and B molecules on their red blood cell surfaces, and type O individuals express neither A nor B. To look at the molecules more closely, focus in at the top panel for just a moment. Each of these different shapes represents a particular type of carbohydrate, or sugar, molecule. The first thing that's done is to assemble a core structure. If nothing else is done to the core structure, that person will be type O. Type A individuals contain an enzyme that modifies the end of the core structure by adding a single carbohydrate to the end, right here. Type B individuals contain a different enzyme that adds a different carbohydrate to the same position, shown here. For type AB, those persons have both enzymes, core structures are modified to both A and B.

**Forward typing methods and the Shroud**

A couple of different approaches were used for forward typing studies, including immunohistochemistry which we've already talked about. The overall results were similar, so we'll focus on those experiments. In these studies, white and bloodstained fibers were mixed with labeled antibodies against the A molecule or B molecule, etc. The samples were washed to remove unbound antibody and evaluated microscopically. In addition, similar experiments were done using labeled irrelevant (negative control) antibodies. In these studies, bloodstained fibers were positive for both A and B molecules in approximately equal intensities, and were negative for reactivity with anti-O antibody. Control (white) fibers were negative for reactivity with all antibodies and bloodstained fibers did not react with irrelevant (negative control) antibodies. Assignment of an AB blood type based on forward typing methods was reported by Baima Bollone in the early 1980s. He would repeat these studies several years later using more specific antibodies and obtain similar results. Some limited typing studies were also reported by Garza-Valdes in 1999.

In discussion of these results, it is sometimes mentioned that their significance is unclear as "a lot of old material types as AB". A few comments related to this. Where this is coming from in relation to forward typing involves so-called "false positives". The ABO antigens are not unique to human red blood cells, in fact, they're not even unique to humans. These molecules are found in many different species, including bacteria, fungi, and insects to name a few. First, not all old blood types

as AB. In studies involving King Tut using similar serological techniques, a blood type of A was reported. Sometimes old blood types as AB, sometimes it doesn't. Regarding the Shroud, it was reported that fibers at the bed of the bloodstain, right next to where the bloodstain ended, tested negative for reactivity with anti-A or anti-B antibodies. If the AB results were simply due to contamination, it is a little hard to imagine that this would be so compartmentalized and not spread out to at least the margins, although this is certainly debatable.

**Reverse typing methods and the Shroud**

So, do we really know that the blood is AB? In a clinical situation involving a blood transfusion, blood type is not evaluated only using forward typing methods. Reverse typing, which measures the presence or absence of specific antibodies in the serum, is also used as this provides a complementary answer, a type of one-two punch. To set this up, I'd like to refer to the fleece experiment performed by Gideon in 1162 B.C. Gideon believed that God was speaking to him but wasn't completely sure. So, he asked God if he could do an experiment, to place a fleece or animal skin on the ground overnight. If God were really behind this, he asked that the fleece be wet the next morning, but the ground around it be completely dry. When Gideon went out the next morning, this is exactly what he found. He felt very confident about the results, until later that afternoon, when he started to second guess himself and have doubts. So, he approached God again and said, "I don't mean to test your patience, but I'd like to do a follow up. This time, let me put the fleece down and the next morning have the fleece be dry, but the ground around it be completely wet." The next day he went out and this was the result. He now had complementary tests that supported each other and went forward very confidently in the results, that God was speaking to him.

This is how forward typing and reverse typing tests work. They complement each other, and provide a type of one-two punch. In reverse typing of the ABO system, a person will have in their serum antibodies against the molecules that they themselves do not express. For example, a person who is type A will have anti-B antibodies in their serum because they themselves do not express B molecules. A person who is type B will have anti-A antibodies present. A person who is type O will have antibodies against both A and B. And type AB, such as reported for the Shroud, will have neither anti-A nor anti-B antibodies present. So in this latter instance we are essentially looking for a negative result.

For fresh blood, such tests are relatively straightforward and work very well. With aged samples, however, such tests can be somewhat problematic in that over time, antibodies can undergo degradation or loss of function. For an antibody to function properly, it is very important that the proper three-dimensional conformation is maintained, that it is correctly folded. Such reverse typing tests depend on an antibody being

turn-key ready (functional), it is not enough for an antibody to simply be present (in a nonfunctional capacity). An assignment of AB type via reverse typing of Shroud samples was reported by Baima Bollone in the early 1980s; however, such results are inconclusive at best. In type AB, you are looking for the absence of antibodies anyway, so the argument becomes rather circular. This is another part of the "all old blood types as AB" statement, it is especially relevant to reverse typing. Thus, in relation to blood typing of the Shroud, only one half of the fleece experiment is present, it lacks a one-two punch. One approach which could possibly provide complementary information is DNA analysis. Unlike forward typing serological methods, which evaluate the A and B molecules on the surfaces of red blood cells, DNA techniques assess the enzymes that are responsible for modifying the core structure into A or B forms. So, can we know that the blood is really AB? Forward typing results suggest yes, but a second confirmatory test is important, many would say essential.

**Other RBC molecules: the MNS system**

Next we'll turn our attention to other red blood cell surface markers. The ABO system is one of many present on red blood cells-it is the most studied because of its important in blood transfusion. I'd like to talk briefly about another system termed MNS. The MN antigens are expressed on red blood cells of anthropoid apes as well as humans. In contrast, the S antigens are specific for human red blood cells only. Shroud fibers were previously evaluated for MNS expression, although the relationship between expression in various types of primates was unknown at the time. These studies showed that unstained (white) control fibers from the Shroud were MNS negative. Bloodstained fibers from the Shroud were positive for both M and N, and most importantly, also for S. These results were reported by Baima Bollone in the mid-1980s. However, the exact significance of these studies is somewhat unclear: they were published in a very brief report and the antibody reactivity for anti-S was described as "fairly good binding". This would be something that would be very interesting to follow up on using more modern reagents.

It's important to keep in mind that for most of the previous studies that were performed polyclonal antibodies were used. Poly, meaning "many", refers to the fact that such antibodies are a mixture, originating from many different clones, each antibody product being slightly different. In the mid-1990s, so-called monoclonal antibody technology was developed, mono meaning "one", from a single clone. Thus, every single monoclonal antibody is derived from a single cell and is exactly identical to the next. Polyclonal antibodies are good. Monoclonal antibodies are extremely good. In 2010, highly specific monoclonal antibodies were developed that can effectively distinguish human blood from that of even closely related primates. Potentially such reagents could be used to definitely

demonstrate that the blood on the Shroud is of human origin. To summarize: studies indicate that the blood is at least primate, the MNS results suggest possibly human. It is unknown if blood from other species (non-primates) may be additionally present. The forward typing studies suggest type AB, although a second confirmatory test, using a different approach, is important.

**Molecular biology (DNA) studies and the Shroud**

This brings us to questions such as: is the blood from a human male? Is it possible to know if the blood is representative of a single individual? Human DNA has been isolated from bloodstained fibers of Shroud, reported by Canale in the mid 1990s, and most famously by Garza-Valdes in the late 1990s, in his book "The DNA of God". More recently, from the Shroud conference in Bari last month, it was revealed that plant and human DNA has been isolated from dust particles that were vacuumed from the Shroud. Regarding the Garza-Valdes studies, portions of three genes were cloned and sequenced: the betaglobin gene, which is a subunit of hemoglobin, and segments of the X chromosome and the Y chromosome. These latter two suggest that the blood may be from a human male. One of the main issues in DNA studies, however, is that of contamination. It can often be difficult to tell if the DNA signal is truly coming from the sample being studied, the bloodstains in this case, or if it simply results from handling of the artifact by various individuals. The average person, in as single day, sheds approximately 400,000 skin cells, some of which contain DNA that can be transferred, called contaminating DNA or touch DNA. There is nothing about any of the three gene segments that were studied that is specific for blood cells. These three genes are expressed in essentially all cell types in the body, including skin cells. One can conclude that human DNA is found on the Shroud, but that is really as far as you can take it: that human DNA has been found on the Shroud. It is unknown if the DNA signal truly originates from blood cells. If the signal were, in fact, coming from blood cells, it would have to be coming from the white blood cells and not red blood cells, as red blood cells in the human lack a nucleus, they are devoid of DNA. One might be able to take advantage of this in that white blood cells (lymphocytes) are unique among all other cells in the body in that they undergo DNA rearrangement. Specific receptor genes are rearranged, cut and spliced, as part of their normal differentiation and development. One could potentially use such an approach (analysis of gene rearrangement) to directly tie in a DNA signal that originates from blood cells on the Shroud.

**DNA degradation and the Shroud**

In addition to the three gene segments reported by Garza-Valdes, is it possible to analyze additional DNA segments? In discussions about DNA and the Shroud it is not uncommon to hear things like, "portions of the X

and Y chromosome have been found, but the rest of the DNA is really too degraded to be useful…". This then begs the question, if the DNA is so degraded in the first place, how were these particular genes isolated in the first place? Did the investigators sift through the cellular debris and rubble and these three genes just happened to survive? This is simply not the case. There is sometimes a misunderstanding as to exactly how such studies were done. The human genome consists of approximately 20,000-30,000 genes. These are three of those genes. These three genes were able to be studied, not because they were leftovers and happened to survive, but, rather, because they were specifically chosen and targeted for study. This was accomplished by using a technique known as the polymerase chain reaction, or PCR for short. The way that this method works is you take a sample, which contains a myriad of different genes, and add in a specific probe that will bind to the particular target gene sequence of interest. The probe will find that gene sequence among all of the other gene sequences that are present. As a result, the target gene of interest will be selectively amplified so that sufficient amounts of material are available for study. One could take the same sample, add in a different probe to a different gene, and amplify that particular gene up. The PCR technique is so efficient that approximately one billion copies of a gene may be generated in just about three hours time. The three genes were not studied by Garza-Valdes because they simply happened to be all that was left and everything else was degraded: they were specifically chosen. So, is the DNA too degraded for (further) analysis? It could be. One would certainly expect that degradation could be an issue given the reported age range (700-2,000 years old) of the sample. This then leads to the question that if degradation is such an issue, where is the DNA signal coming from? On the other hand, even if degraded/fragmented to a certain extent, modern DNA analysis is doing some impressive things with very small bits of DNA isolated from very ancient, very degraded samples.

**Development of a Shroud CODIS: Nuclear and mitochondrial DNA**

Modern DNA analysis has the potential to evaluate what type of heterogeneity might exist, on a molecular level, within the bloodstains. Specifically, it might be used to determine if the DNA pattern is consistent with the blood being from a single individual. For these types of studies, there are two types of DNA to consider: nuclear DNA, which comprises the bulk of the human genome and exists as two copies per cell, and mitochondrial DNA. Mitochondria are an organelle within the cell that exist to provide energy for cellular functions. Mitochondria have their own genome (set of genes), distinct from those in the nucleus, which consists of only thirty seven total genes, existing in the form of a loop. One mitochondria can contain multiple copies of DNA, and multiple mitochondria may exist within a single cell, resulting in the copy number being much higher than nuclear DNA. Depending upon the

particular cell type, approximately one hundred to ten thousand copies of mitochondrial DNA may exist per cell. Thus, it is often a major consideration for study in cases involving aged samples where degradation is expected to be a major issue. Unlike nuclear DNA, which is inherited from both parents, one copy from mom and one copy from dad, mitochondrial DNA is maternal in origin, that is, inherited solely from the mother. Thus, the chances of two unrelated individuals sharing the same mitochondrial DNA profile are extreme remote. Because of its unique maternal inheritance and especially the high copy number, analysis of mitochondrial DNA is often favored in studies involving ancient samples with fragmented/degraded DNA. Concerning DNA heterogeneity, there are certain DNA segments (genes, non-coding segments) that are identical among all individuals. There are also certain segments that are not; these are the most helpful in sorting out heterogeneity issues. For a given gene, nuclear DNA can be expressed as 2 forms max, one inherited from mom, one from dad. For mitochondrial DNA this is one form. Thus, if multiple forms of a particular gene are present in a sample that exceed the maximum, this in an indication that contamination (heterogeneity) is present.

To evaluate the extent to which heterogeneity might exist among the bloodstains is the idea behind the development of a Shroud CODIS, or Combined Data Index System. This is the terminology that the FBI uses for their DNA database and simply refers to a systematic analysis to catalog and compare particular DNA sequences of interest. For the Shroud, this would involve sampling multiple bloodstains and determining if a consensus sequence pattern could be observed. By extension such analysis could also be expanded to comparison with DNA sequences present on other artifacts, such as the Sudarium of Oviedo. Finally, one of the ultimate questions that many would like to know is exactly whose blood is it? That is, is there any way that DNA analysis could tell you exactly who the man of the Shroud is, i.e. whether it is Jesus? The answer is no. DNA studies are by nature comparative, such a conclusion would require that a DNA profile of Jesus was available on file to compare the sample results to. As no DNA profile of Jesus exists on record, this simply could not be done. Relatedly, there are over thirty plus books available from amazon that have cloning Jesus from the Shroud bloodstains as a storyline; there are two movies and even a comic book series. Cloning the man on the Shroud would involve at a minimum an intact human genome of 20,000-30,000 genes; the aforementioned studies would at best look at just a few of such genes. Such cloning scenarios are well within the area of science fiction (for even other, additional reasons): you can rest easy on this one.

In summary, DNA studies have the potential to distinguish if the bloodstains are representative of a single or multiple individual(s) at the molecular level. Additionally, a molecular biology approach could

potentially be used to demonstrate that a DNA signal is present on the Shroud that originates from blood cells. Thank you.